Supporting Online Material

Materials and Methods

Drosophila stocks

Oregon R (*OR*) or *w118* lines were used as wild-type controls. The following deficiency stocks were used: *CG4225*: *Df(3R)Exel8162* (deletes 10 genes) , *mdr65*: *Df(3L)Exel6108* (deletes 6 genes), *mdr49*: *Df(2R)Exel7123* (deletes 17 genes), *mdr50*: *Df(2R)Exel7131* (deletes 18 genes), *CG7955*: *Df(3L)ED4283* (unknown number of genes deleted), *sras*: *Df(3L)Exel6105* (deletes 24 genes), *CG11268*: *Df(3L)ED4486* (deletes 60 genes), *Gγ1*: *Df(2R)H3E1* (deletes 55 genes), *CG9000/CG9001/CG9002*: *Df(2R)Exel6065* (deletes 41 genes). The following P-element stocks were used: $mdr49^{KG08661}$, $mdr65^{KG08723}$, $CG3156^{f03674}$, $Sras^{KG04579}$, $Gγ1^{K08017}$ (Bloomington Stock Center). Of these, P-element insertion KG08661 is located 1.6kb upstream of the gene start of *mrd49*, KG08723 is located in the coding region of *mdr65* and the remaining are located in the 5' UTR of the respective genes (Flybase). Other stocks used were: *twi-Gal4* (*S1*), *elav-Gal4 UAShmgcr* (constructed by M. Van Doren), enhancer trap line *68-77* (*S2*), P_{nos}*::egfp-moe::nos* 3'UTR (*S3*).

UASmdr49 and *UASSTE6-GFP* lines were constructed by cloning the corresponding cDNAs into pUAST, followed by standard P-element mediated transformation techniques. The cDNA for *STE6-GFP* was kindly provided by Susan Michaelis (*S4*).

To generate *UASmdr49* KpnI and BglII restriction sites were added to the ends of the cDNA by PCR and cloned into pUAST (*S1*). To generate *UASSTE6-GFP*, the cDNA clone was digested with EagI and NotI and ligated into the NotI/EagI site of pUAST. For both transgenes, the constructs were sequenced and displayed no errors.

Generation and characterization of the mdr49 mutation.

The $mdr49^{KG08661}$ P-element insertion was mobilized by crossing to the transposase stock *w; Sp/CyO; Δ2-3Sb/TM6Ubx*. 93 independent excision lines were generated and all were further characterized by PCR with different sets of flanking primers. All larger deletions were also stained for Vasa to assess the germ cell migration phenotype. The allele

described is a 3.4kb deletion and its breakpoints were defined by sequencing of the PCR product.

In situ hybridization and immunohistochemistry

In situ hybridizations were performed as described in *S5*. The RNA probes were constructed from cDNA clones from the Drosophila Genomics Resource Center for each gene: *mdr49* (SD10012), *mdr65* (RE14657) and *CG7955* (GH20617) or from genomic DNA in the case of *mdr50* by transcription with T3, T7 or SP6 polymerase and using the DIG RNA labeling kit (Roche). Probe hybridization was visualized using an alkaline-phosphatase conjugated anti-DIG antibody (Roche) followed by treatment with NBT and BCIP.

Embryos were fixed in 4% formaldehyde and stained using standard protocols and the following antibodies: rabbit anti-Vasa (1/10,000, made by A.Williamson and H. Zinszner, Lehmann lab), mouse anti-β-Galactosidase (1/1000, Promega), mouse anti-Actin (1/100, A4700-Sigma). Antibody detection was done using anti-mouse and anti-rabbit_biotinylated secondary antibodies (1/500, Jackson ImmunoResearch). For anti-actin the detection was done using an anti-mouse Cy3 conjugated secondary antibody (1/500, Jackson ImunoResearch). Embryos stained with DAB were mounted in Epon and viewed on a Zeiss Axioskop microscope. Mutant embryos were compared either to a wild-type control or to non-mutant siblings in the same experiment; the control siblings were distinguished from the experimental embryos by a balancer chromosome marked with a LacZ transgene. Student t-tests were used to assess significance in germ cell numbers and p values of less than 0.01 were considered significant. All embryo collections were performed at room temperature.

RT-PCR

To assess the mRNA levels of $mdr49^{KG08661}$ and $mdr49^{\Delta 3.16}$, embryos from overnight collections were used to extract total RNA using TRIzol reagent (Invitrogen). Reverse transcription (RT) reactions were performed using Superscript II (Invitrogen) following the manufacturer's protocol. PCR amplification was performed for 26 cycles for the *mdr49* alleles.

To assess downregulation of gene expression after RNAi treatment for *hmgcr*, *mdr49*, *βGGTI* and *syx5*, RNA was extracted from approximately $1x10^7$ cells per each experiment at least twice using TRIzol reagent (Invitrogen). As above, reverse transcription (RT) reactions were performed using Superscript II (Invitrogen) following the manufacturer's protocol. PCR amplification was performed for 25 cycles for all genes, except for *βGGTI*, in which 30 cycles were done. Primers were chosen for non-overlapping regions of dsRNA. Sequences of all primers are given upon request.

FACS sorting of germ cells

P_{nos}*::egfp-moe::nos* 3'UTR embryos from a 10-11 hrs collection were collected in large cages, dechorionated and mashed using a glass pestle and dissociation buffer (*S6*). The cell suspension was passed first through a 100μm and then a 22μm filter to further dissociate the cell suspension into single cells and doublets. Germ cells were subsequently FACS sorted (MoFlo cell sorter) into Schneider's media, with the gating made for the highest GFP level and smaller cell size to select for only single germ cells. To confirm the homogeneity and integrity of the germ cell population, these were visualized in a fluorescence microscope (Leica DM-RXA). To determine that sorted cells were indeed germ cells they were stained with Vasa antibody.

Transwell migration assay

Serum-free conditioned media was collected from transfected or treated Kc cells. Conditioned media was spun 1x for 10 min at 1000rpm and then again for 40min at 4000rpm (Eppendorf Centrifuge 5810) and equal quantities added to the bottom wells of a 96 well chemotaxis chamber plate (ChemoTx Disposable Chemotaxis Chamber with a 10μm filter pore size, Neuroprobe), according to the manufacture's instructions. Approximately 3000 sorted germ cells were added directly to the top of each well. The chemotaxis chamber was returned to the incubator at 25^{o}C and incubated for 1 hr to allow migration. The 1 hr optimal incubation time was defined experimentally as the point at which directed migration is maximum and random migration is at its minimum, as suggested by the manufacturer. After incubation cells that had not migrated were wiped off the top wells and the plates were spun at 460g for 10min. All germ cells in the lower

chamber were collected into lab TEK chambers (Nalge Nunc) for manual counting in an inverted confocal microscope (Zeiss LSM-510). Student t-tests were used to assess significance in germ cell migration across experiments and p-values of less than 0.05 were considered significant

Transfections

The following cDNAs were used for transfections in 60mmm dishes: UAShmgcr (*S7*) UASmdr49 (this study) in co-transfections with act-Gal4VP16 (a gift from Jessica Treisman). Kc cells were transfected using the Effectene transfection reagent (Qiagen).

Cell stainings

Kc cells were stained in 4% fresh paraformaldehyde in small round coverslips, using standard procedures. anti-Vasa was detected using an anti-rabbit Cy3 conjugated secondary antibody (1/500, Jackson ImunoResearch), anti-GFP (1/100, JL8-Clontech) was detected using an anti-mouse Alexa Fluor 488 (1/500, Jackson ImunoResearch).

RNAi

Kc cells in 60mm dishes were incubated with 30μg of dsRNA for 3 days as described elsewhere (*S8*). dsRNAs were constructed as described elsewhere (Drosophila RNAi Screening Center: http://flyrnai.org). Primers for dsRNA construction are given upon request. In all cases cell viability or growth was not affected as confirmed by cell counting after the 3-day incubation period.

Supporting Text

Characterization of *mdr49*^{KG08611} and *mdr49*$^{\Delta 3.16}$

The P-element allele *mdr49*^{KG08611} is located 1.6Kb upstream of the predicted *mdr49* transcription start (Fig S1A). However this allele is not an RNA null (Fig S1B, S1C). *mdr49*^{KG08611} mutant embryos have a weak germ cell migration phenotype in which about six germ cells failed to associate with SGP cells (Fig S1D, Table S2 and Fig 1C). We generated a deletion allele, *mdr49*$^{\Delta 3.16}$, through imprecise excision of the P-element KG08611 (Fig S1A). RT-PCR from *mdr49*$^{\Delta 3.16}$ mutant embryos revealed a significant reduction in *mdr49* RNA levels (Fig 1B). This phenotype was only slightly weaker than the phenotype of embryos carrying a deficiency *Df(2R)Exel7123* (*Df(2R)mdr49*) either in trans to *mdr49*$^{\Delta 3.16}$ or homozygous (Table S2). Thus, *mdr49*$^{\Delta 3.16}$ is a strong loss of function allele.

Genomic search of post-prenylation processing enzymes

A cluster of three genes *CG9000/CG9001/CG9002* encodes the predicted orthologs of the yeast Ste24p prenyl protease type I; we refer to these as Dmel\Ste24. Of these CG9000 shares the highest degree of homology (Table S4 and Fig S2). A single gene, *Sras*, is the predicted Drosophila ortholog of the yeast prenyl protease type II encoded by *RCE1* (Table S4). Drosophila CG11268 is the predicted ortholog of Ste14/ICMT isoprenylcysteine carboxylmethyltransferase; we refer to this as Dmel\Ste14 (Table S4 and Fig S2).

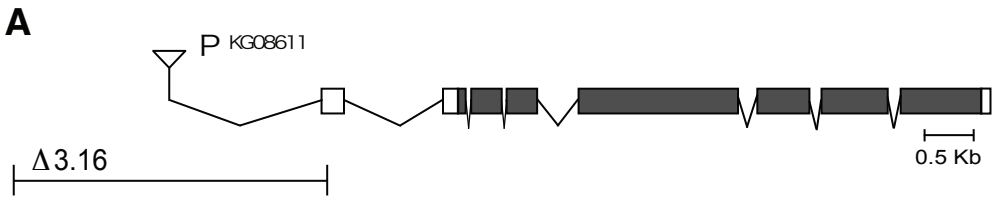
The prenyl protease type II Sras is not required for germ cell migration

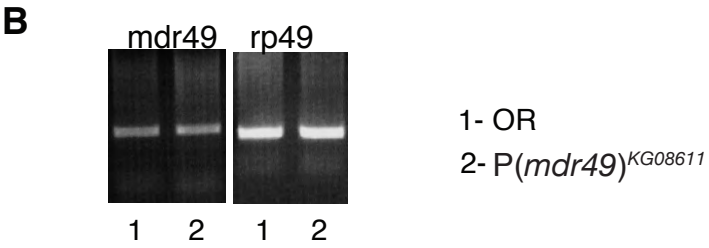
The prenyl protease type I Dmel*ste24* affects germ cell migration but does not affect embryo patterning. This finding indicates that in Drosophila other prenylated proteins essential for development and patterning such as the small GTPases Rho and Rac are likely modified by the type II prenylprotease Sras. As expected, a deletion for *Sras* *Df(3L)Exel6105*, (*Df(3L)Sras*), affects embryonic patterning thereby preventing analysis of the homozygous phenotype. We therefore addressed the role of *Sras* in a suppression

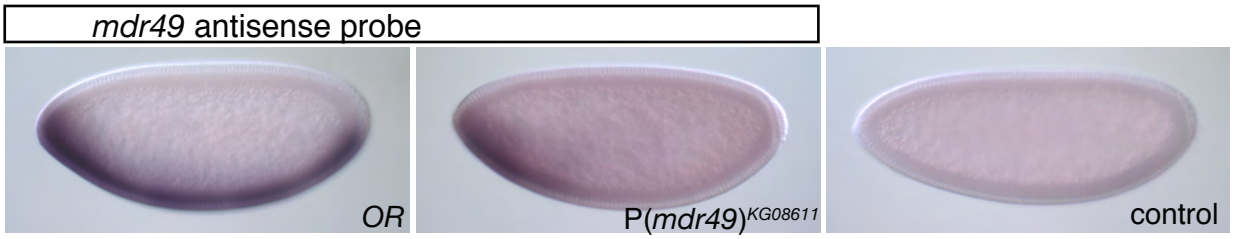
assay similar to that outlined for *mdr49* using this deletion. Removing one copy of *Df(3L)Sras* failed to inhibit the migration of germ cells toward *hmgcr* ectopically expressed in the CNS (Fig S3), suggesting that germ cell migration is specifically dependent on Ste24 type I proteolysis of a putative attractant.

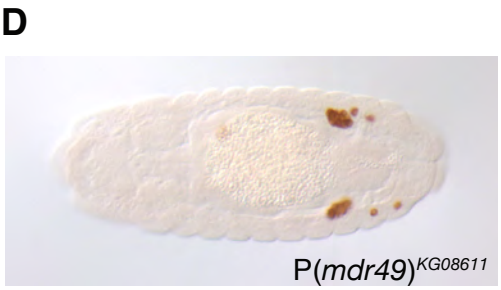
Gγ1 is not required for germ cell migration

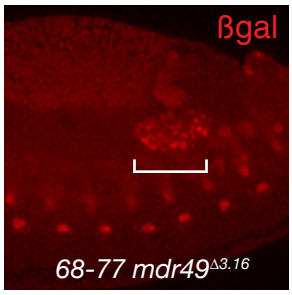
Besides Ras only one other target for protein prenylation has been identified in Drosophila. Prenylation of the small G protein Gγ has been shown to be *hmgcr* dependent and required for heart cell association in Drosophila (*S9*). We do not believe that prenylation of Gγ is required for germ cell attraction, since embryos zygotically mutant for *Gγ* do not show a germ cell migration phenotype (Fig S6). Moreover, *sar1,* which encodes a protein required for budding of vesicles from ER to the Golgi (*S10*), causes a similar heart defect as *Gγ* (*S9*), suggesting that prenylation of Gγ activates an internal cell signaling process that via the indirect pathway leads to constitutive secretion of a heart cell attractant.

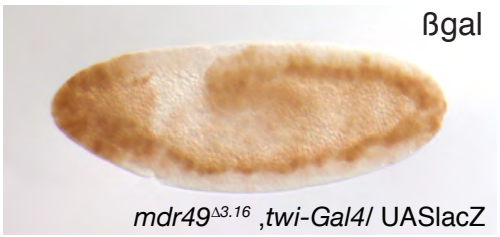




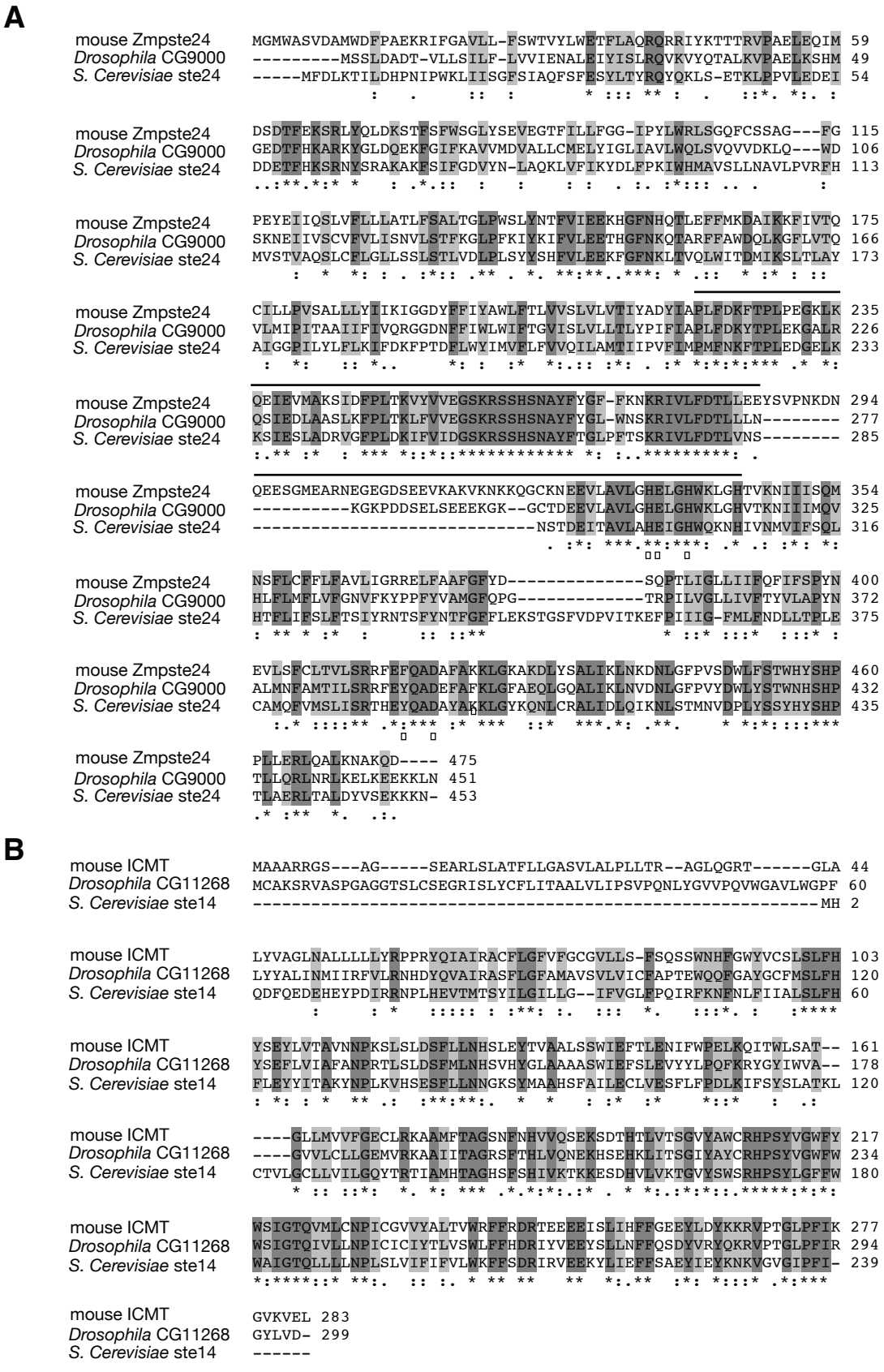




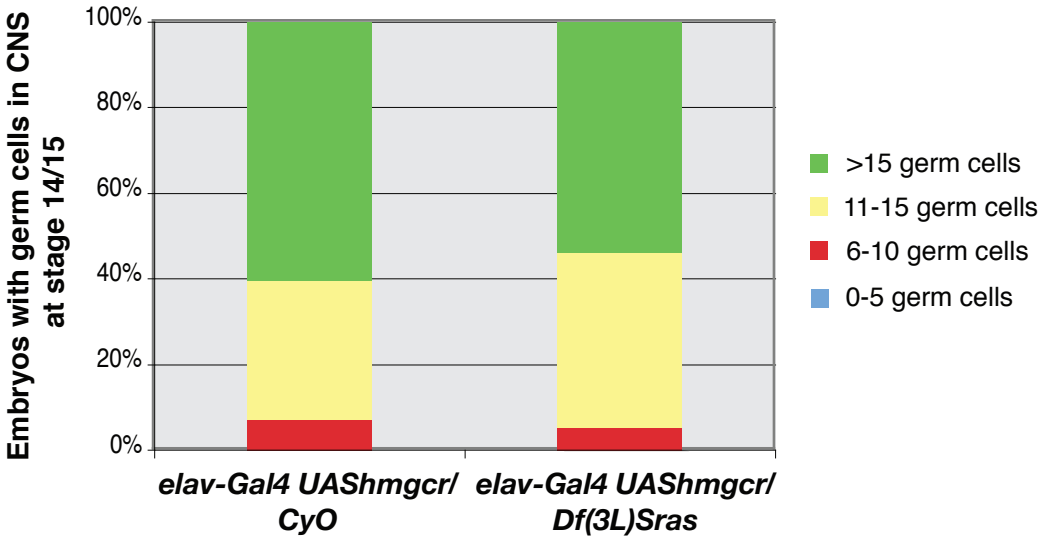




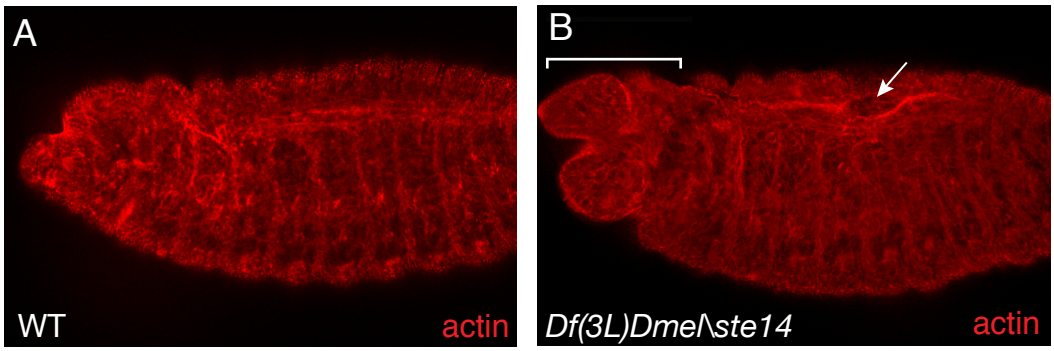
Supporting Figure 1
(A) P element insertion site and deletion in *mdr49* in Drosophila genomic DNA. Exons are represented by black boxes and white boxes correspond to untranslated regions.
The imprecise excision line Δ3.16 is represented denoting the extent of the deletion.
(B) RT-PCR of $mdr49^{KG08611}$ embryos in which RNA is still produced, (rp49 as loading control).
(C) In situ hybridization shows reduced mRNA expression in $mdr49^{KG08611}$embryos, sense probe is used as control. (D) Germ cells (brown) are lost in the posterior mesoderm in $mdr49^{KG08611}$ embryos (stage 14, posterior to right). (E) The gonad is normally specified in $mdr49^{\Delta 3.16}$ mutation; β-gal staining of mutant embryos that carry LacZ insertion line *68-77*, which marks SGPs (bracket)
(F) mesoderm expression driven by mesodermal driver in $mdr49^{\Delta 3.16}$, *twi-Gal4* recombinant line, as seen by β-gal staining (brown) in stage 10 embryos (posterior to right, dorsal up)



Supporting Figure 2
Sequence alignment of prenylproteases type I (A) and isoprenylcysteine carboxylmethyltransferases (B) in mouse, yeast (*Saccharomyces cerevisiae*) and *Drosophila melanogaster*. Identical residues in all sequences are highlighted in dark grey and represented by " * ", conserved substitutions are highlighted in light grey represented by " : " and semi-conserved substitutions are represented by " . ". Predicted catalytic and active sites are represented by open squares. Black lines above sequences represent predicted cytoplasmic domains.

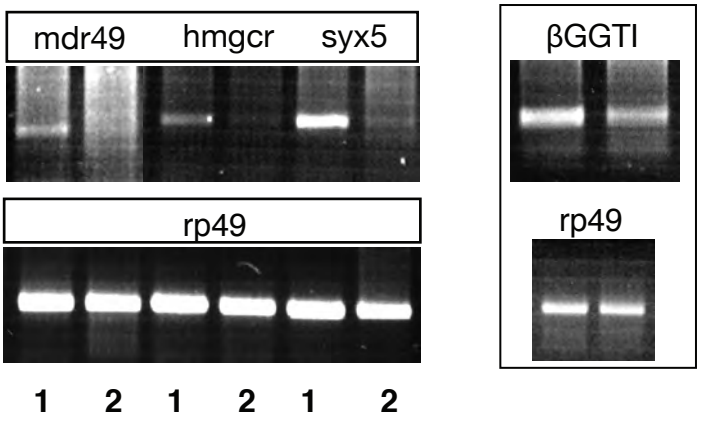


Supporting Figure 3
Prenyl protease type II, Sras, is not required for germ cell migration.
A deficiency for *Sras* can not suppress ectopic germ cell migration to the CNS after misexpression of *hmgcr*.

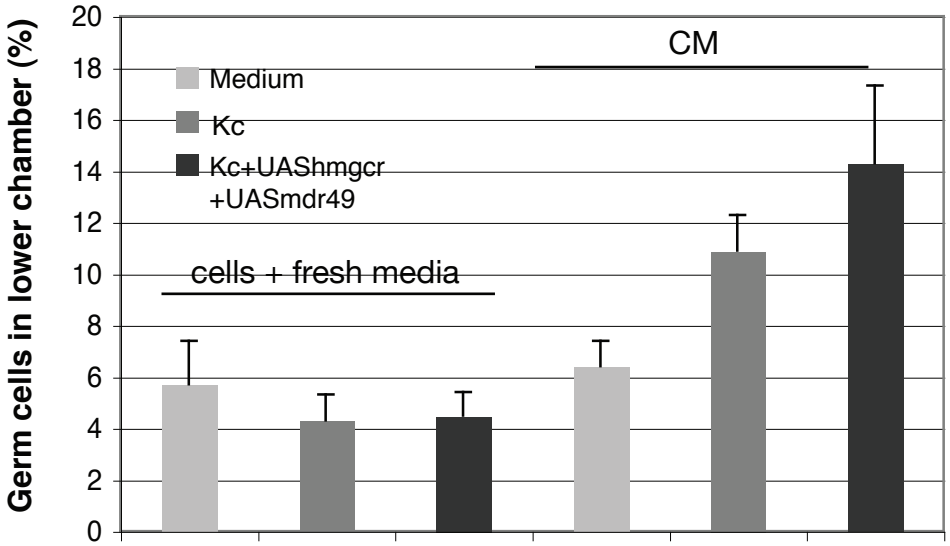


Supporting Figure 4
Embryos deficient for Dmel*ste14* (isoprenylcysteine caboxylmethyltransferase) show head involution (bracket) and mild dorsal closure (arrow) defects (B) as compared to wild-type embryos at the same stage (A) (posterior to the right).

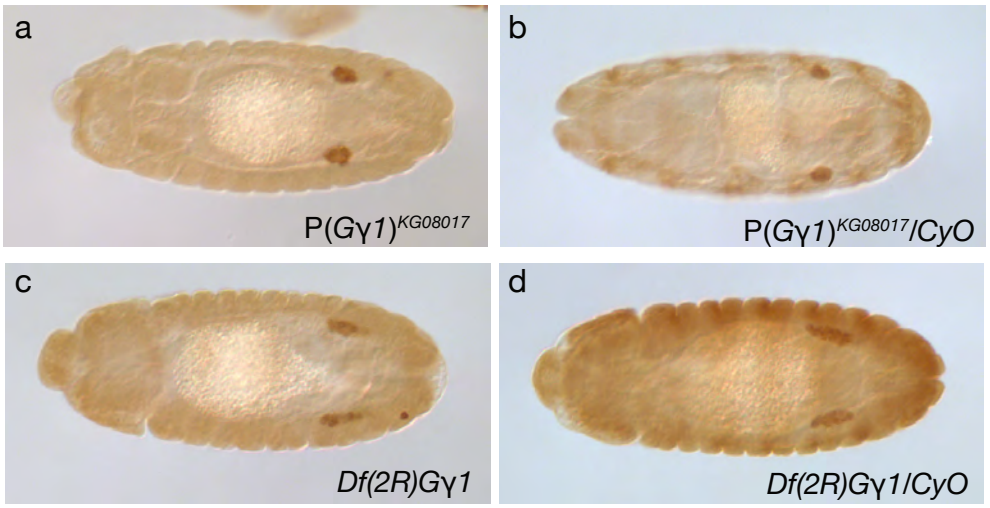
A

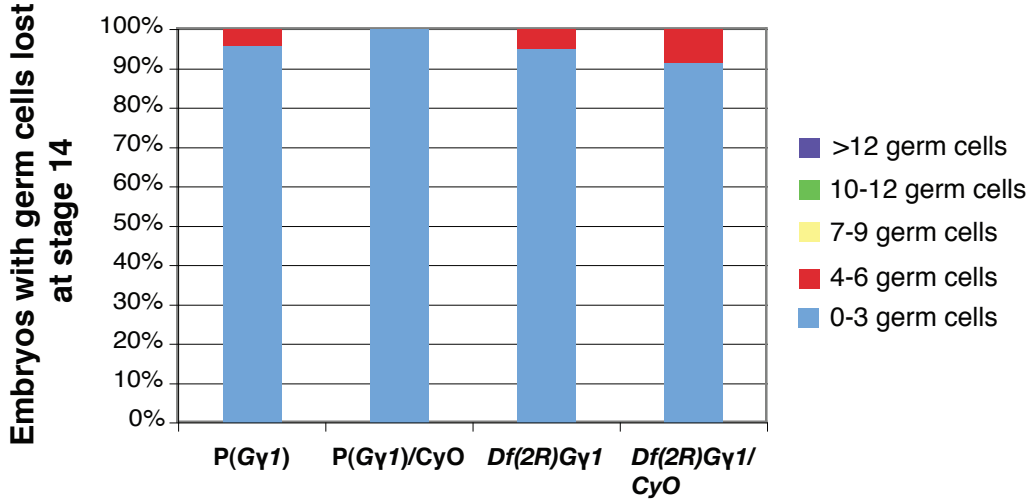


B



Supporting Figure 5
(A) RT-PCR of all genes tested in Kc cells with or without dsRNA treatment (1-Kc ; 2- Kc + dsRNA). rp49 is used as a loading control and the genes tested are indicated on top of each gel. For all genes PCR amplification was done for 26 cycles, except for βGGTI in which PCR amplification was performed for 30 cycles. Experiment repeated 2 times, in duplicate.
(B) Germ cells migrate towards conditioned media alone but not towards expressing cells when conditioned media was substituted by fresh media, indicating that the signal is secreted. Experiment repeated 2 times, in triplicate.





Supporting Figure 6
The small G protein Gγ1 is not required for germ cell migration.
Embryos mutant for Gγ1 (a) or carrying a deficiency for this gene (c)
have wild-type germ cell migration as compared to sibling controls (b and d).
Embryos are at stages 14-16 and oriented posterior to the right.
A quantification of the phenotype is shown below.
(P(*Gγ1*): n=47; P(*Gγ1*)/*CyO*: n=51; *Df(2R)Gγ1*: n=20; *Df(2R)Gγ1/CyO*: n=47)

Supporting Table 1

ABCB genes in Drosophila tested for mRNA expression and germ cell migration phenotype during embryogenesis. ∗ and ∗∗ indicate mRNA expression patterns previously shown by BDGP and fly-FISH respectively (www.fruitfly.org; www.utoronto.ca/krause). WT: wild type, ND: not determined, because not expressed in embryo. ND◊: no zygotic phenotype and maternal effect phenotype not determined because of recessive lethality.

Gene	**mRNA Expression**	**Migration phenotype**	**Suppression of hmgcr dependent migration to CNS**
CG10226	Maternal ∗	WT	no
mdr65	Glial cells in CNS	WT	no
mdr49	**Mesoderm**	**Germ cell migration to SGPs affected**	**yes**
mdr50	Maternal and ubiquitous	WT	no
CG1824	Not expressed in the embryo ∗	ND	ND
CG4225	Maternal ∗∗	ND ◊	ND ◊
CG7955	Maternal ∗	WT	no
CG3156	Maternal ∗∗	WT	no

Supporting Table 2
Average number of germ cells in embryos of indicated genotypes. SD-standard deviation, p-value compared to phenotype of embryos mutant for precise excision of *mdr49*^{KG08611}(revertant) for *mdr49*$^{\Delta 3.16}$ genotype and to WT (wild type) for all other *mdr49* genotypes. p-value of Dmel*ste24* deficiency as compared to heterozygous sibling. N: number of embryos analyzed.

	Average number of germ cells lost at stage 14	**p-value**	**N**
WT (*Oregon R*)	1.61 ± 1.5 SD	_	157
mdr49^{KG08611} Revertant	2.19 ± 1.7 SD	_	32
Df(2R)mdr49	5.56 ± 2.7 SD	p< 0.0001	86
P(*mdr49*) ^{KG08661}	3.28 ± 2.3 SD	p<0.0001	165
Df(2R)mdr49/ P(*mdr49*)^{KG08661}	3.63 ± 2.1 SD	p<0.0001	85
mdr49$^{\Delta 3.16}$*/CyO*	3.03 ± 2.1 SD	0.088	32
mdr49$^{\Delta 3.16}$	4.69 ± 2.6 SD	p<0.0001	105
Df(2R)mdr49/ *mdr49*$^{\Delta 3.16}$	4.82 ± 2.8 SD	p<0.0001	57
Df(2R)Dmel\ste24/CyO	2.3 ± 2.1 SD	_	92
Df(2R)Dmel\ste24	6 ± 3.1 SD	1.3x 10^{-15}	67

Supporting Table 3
Average number of germ cells in embryos of indicated genotypes. SD: standard deviation, p-value as compared to control (*elav-Gal4 UAShmgcr*), N: number of embryos analyzed.

	Average number of germ cells in CNS at stage 13	**p-value**	**N**
elav-Gal4 UAShmgcr/+	5.5 ± 2.5 SD	_	55
elav-Gal4 UAShmgcr/ Df(2R)mdr49	3.2 ± 2.5 SD	6.3×10^{-7}	65
elav-Gal4 UAShmgcr/ P(*mdr49*)^{KG08661}	2.7 ± 1.7 SD	3.5×10^{-8}	34

Supporting Table 4
Predicted post-prenylation processing enzymes in Drosophila compared to mating yeast (*S. cerevisiae*) and mammalian orthologues

	S. cerevisiae	**Drosophila**	**Mammals**
Prenyl proteolysis	Rce1p- cleaves Ras and can also cleave a-factor	Sras (Rce1 like)	Rce1- cleaves Ras (*S11*)
	Ste24p- cleaves a-factor	CG9000 CG9001 CG9002 (Dmel\Ste24)	Zmpste24- cleaves Lamin a (*S12*)
Carboxymethylation	Ste14p	CG11268 (Dmel\Ste14)	ICMT

Supporting Table 5
Average number of germ cells in embryos of indicated genotypes. SD: standard deviation, p-value as compared to control (*elav-Gal4 UAShmgcr*), N: number of embryos analyzed.

	Average number of germ cells in ectopic locations at stage 14	**p-value**	**N**
elav-Gal4 UAShmgcr/+	11.6 ± 3.4 SD	_	38
elav-Gal4 UAShmgcr/ Df(3L)Dmel\ste14	8.4 ± 2.8 SD	0.000015	44
elav-Gal4 UAShmgcr/ CyO	15.976 ± 3.47 SD	_	43
elav-Gal4 UAShmgcr/ Df(3L)Sras	15.564 ± 3.08 SD	0.57	39

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